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Aminoglycoside Resistance and Susceptibility Testing Errors in *Acinetobacter baumannii-calcoaceticus* Complex▽

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Antimicrobial resistance is depleting the pharmacopeia of agents clinically useful against Gram-negative bacilli. As the number of active agents diminishes, accurate susceptibility testing becomes critical. We studied the susceptibilities of 107 isolates of the *Acinetobacter baumannii-calcoaceticus* complex to amikacin, gentamicin, and tobramycin using disk diffusion, Etest, as well as the Phoenix, Vitek 2, and MicroScan automated systems, and compared the results to those obtained by broth microdilution. Genes encoding aminoglycoside-modifying enzymes (AMEs) were detected by multiplex PCR, and clonal relationships were determined by pulsed-field gel electrophoresis. Tobramycin was the most active aminoglycoside (27.1% of isolates were susceptible). Disk diffusion and Etest tended to be more accurate than the Vitek 2, Phoenix, and MicroScan automated systems; but errors were noted with all methods. The Vitek 2 instrument incorrectly reported that more than one-third of the isolates were susceptible to amikacin (a very major error). Isolates were polyclonal, with 26 distinct strains, and carried multiple AME genes unrelated to the strain type. The presence of the *ant(2″)-Ia* gene was statistically associated with resistance to each aminoglycoside. The AME genotype accounted for the resistance profile observed in a minority of isolates, suggesting the involvement of multiple resistance mechanisms. Hospital pharmacy records indicated the preferential use of amikacin over other aminoglycosides in the burn intensive care unit, where aminoglycoside resistance is prevalent. The resistance in that unit did not correlate with a predominant strain, AME genotype, or total annual aminoglycoside consumption. Susceptibility to tobramycin increased, even though susceptible isolates carried AME genotypes predicting the inactivation of tobramycin. Determination of the relative contribution of multiple concurrent resistance mechanisms may improve our understanding of aminoglycoside resistance in the *Acinetobacter baumannii-calcoaceticus* complex.

Antimicrobial resistance is depleting the pharmacopeia of agents clinically useful against bacterial infections. The problem is particularly acute for Gram-negative bacteria, for which few new pharmaceuticals with activities against these organisms are in development. A select group of organisms are responsible for the bulk of this problem (6). This includes the *Acinetobacter baumannii-calcoaceticus* complex, a group of Gram-negative bacteria adept at demonstrating resistance to multiple agents.

As the number of active agents is reduced, prescribing patterns shift toward antimicrobials with a greater potential for toxicity, such as colistimethate and the aminoglycosides. Increased antimicrobial use has been associated with higher rates of resistance (7, 20), and the antibiogram has been noted to parallel changing prescribing patterns (11). The intensified use of a shrinking pool of agents may therefore accelerate the development of resistance to the last active drugs. Optimal susceptibility testing, in concert with surveillance for the most common resistance mechanisms, antimicrobial usage patterns, and phenotypic resistance, may help guide strategies to preserve the activities of the remaining active agents.

The importance of accurate susceptibility testing becomes paramount as the available treatments diminish. Most modern microbiology laboratories rely heavily on automated systems for identification and susceptibility testing. Such systems can give inaccurate results for selected organism-antimicrobial combinations, and nonreporting or confirmation by a manual method is recommended in these cases (2, 3, 29). However, manual testing errors can also occur, as noted in *Acinetobacter* for the tetracyclines (1) and colistin (13, 23). For *Acinetobacter*, we have observed in clinical practice at the San Antonio Military Medical Center results of susceptibility to amikacin from the Vitek 2 automated system (bioMérieux, Durham, NC), whereas the disk diffusion method indicated resistance, a finding also noted by others (3, 15).

We conducted a retrospective study of *A. baumannii-calcoaceticus* complex isolates to investigate whether the accuracy of amikacin susceptibilities reported by the Vitek 2 automated

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system for these organisms might be influenced by the presence of aminoglycoside-modifying enzymes (AMEs), as was recently reported to occur in association with the *armA* 16S rRNA methylase (18). In addition, we sought to assess the accuracies of common testing methods, determine the antibiograms for clinically useful aminoglycosides, and survey the distribution of AME genotypes among the *Acinetobacter* isolates at the San Antonio Military Medical Center. We hypothesized that the high rates of aminoglycoside resistance present in our burn intensive care unit (ICU) correlate with AME genes and would correspond to aminoglycoside usage, as previously observed for *Acinetobacter* (7) and other Gram-negative organisms (20).

MATERIALS AND METHODS

Bacterial isolates. A total of 107 nonrecurring isolates of *Acinetobacter baumannii-calcoaceticus* complex from a blood or a wound infection of a single patient occurring at our facility between 2006 and 2008 were studied, including 45 isolates arising from infections in the burn intensive care unit. The strains were stored in glycerol at −80°C and were passaged twice on blood agar plates (Remel, Lenexa, KS) before they were tested. *A. baumannii* BM2686 (19), *Citrobacter freundii* 971, *Serratia marcescens* 972, and *Enterobacter cloacae* 973 [which contain *aac(6′)-Ih*, *ant(2″)-Ia*, *aac(3)-I*, and *aac(3)-II*, respectively] were used as positive controls in PCR amplifications of the AME genes (kindly provided by Michel Simonet, University of Lille, Lille, France). *Escherichia coli* ATCC 87419 containing *aac(6′)-Ib* and *E. coli* ATCC 87434 containing *aph(3′)-VIa* were obtained commercially (American Type Culture Collection, Manassas, VA).

Bacterial identification. Isolates were identified by using the Vitek 2 (bioMérieux) and Phoenix (Becton Dickinson and Co., Franklin Lakes, NJ) automated microbiology systems. In addition, all isolates were examined by PCR amplification for the presence of the $bla_{\text{OXA-51-like}}$ beta-lactamase gene intrinsic to *A. baumannii*, according to previously published methods (33). Amplified ribosomal DNA restriction analysis (ARDRA) was performed with the restriction enzymes AluI, HinfI, HhaI, RsaI, MboI, and MspI, as described previously (34).

PFGE. Clonal relationships were assessed by pulsed-field gel electrophoresis (PFGE) by the Pulsenet protocol of the Centers for Disease Control and Prevention, with modifications for *Acinetobacter* being used (8). Genomic DNA was prepared with a CHEF bacterial genomic DNA plug kit (Bio-Rad Laboratories, Hercules, CA), digested with ApaI (New England BioLabs, Ipswich, MA) overnight at room temperature, and separated on a 1% SeaKem Gold agarose gel in 0.5× Tris-borate-EDTA buffer. PFGE was performed with a CHEF-DR III system (Bio-Rad Laboratories) and a gradient of 6 V/cm at a 120° angle, with the pulse time increasing from 5 to 20 s. Electrophoresis was run at 14°C for 18.5 h. DNA from *Salmonella enterica* serovar Braenderup ATCC BAA-664 was used as a molecular size standard. The gels were stained with 1 mg/ml ethidium bromide for 30 min, followed by three 30-min cycles of destaining in water. Gel images were captured with a GeneFlash gel documentation system (Syngene, Frederick, MD) and analyzed with BioNumerics software (Applied Maths Inc., Austin, TX). The PFGE patterns were interpreted and grouped into pulsed-field types by using established criteria (32).

Antimicrobial susceptibility testing. The susceptibilities of the isolates and the MICs of gentamicin, tobramycin, and amikacin for the isolates were determined by broth microdilution with cation-adjusted Mueller-Hinton broth and disk diffusion, by use of the methods and interpretive criteria of the CLSI (36, 37), and by Etest (AB Biodisk, Solna, Sweden), according to the manufacturer's instructions. The susceptibilities of all isolates were determined from a single measurement. Manual colony counts were performed to verify the bacterial density of the inoculum solutions used for the manual methods. The isolates also underwent susceptibility testing with the Vitek 2 (bioMérieux), MicroScan WalkAway (Siemens, Deerfield, IL), and Phoenix (Becton Dickinson and Co.) automated systems, according to the manufacturers' instructions. Phoenix ID-123 panels and MicroScan-Neg MIC type 32 panels were used. Due to the modernization of the clinical laboratory, susceptibilities for clinical use had initially been obtained by use of the Vitek GNS 121 card, and for later isolates they were obtained by use of the AST-GN15 card. As tobramycin is not included on the latter card, susceptibilities to tobramycin were determined, when necessary, by using the Vitek AST-GN25 card. The discordance of the results between the reference broth microdilution and the other methods were tabulated as very major errors (susceptibility was reported when the isolate was resistant), major errors (resistance was reported when the isolate was susceptible), or minor errors (intermediate was reported when the isolate was resistant or susceptible, or vice versa) (12).

TABLE 1. Primer sequences used in the study

Primer set	Nucleotide sequence (5′ → 3′)[a]	Target DNA
1a	F: GACATAAGCCTGTTCGGTT R: CCCGCTTTCTCGTAGCA	*aac(3)-Ia*
1b	F: ATGCATACGCGGAAGGC R: TGCTGGCACGATCGGAG	*aac(3)-IIa*
1c	F: TGCCGATATCTGAATC R: ACACCACACGTTCAG	*aac(6′)-Ih*
2a	F: CGGAAACAGCGTTTTAGA R: TTCCTTTTGTCAGGTC	*aph(3′)-VI*
2b	F: ATCTGCCGCTCTGGAT R: CGAGCCTGTAGGACT	*ant(2″)-Ia*
2c	F: GAGGAAGGTGGGGATGACGT R: AGGCCCGGGAACGTATTCAC	*rrn*
3a	F: CGAGCATCAAATGAAACTGC R: GCGTTGCCAATGATGTTACAG	*aph(3′)-Ia*
3b	F: TATGAGTGGCTAAATCGAT R: CCCGCTTTCTCGTAGCA	*aac(6′)-Ib*

[a] F, forward primer; R, reverse primer.

Gene amplification and detection. Three multiplex PCR assays were conducted by a previously described method (27) to identify DNA sequences encoding the aminoglycoside acetyltransferase (AAC), adenyltransferase (ANT), and phosphotransferase (APH) enzymes in *A. baumannii*. Triplex assay 1 included primers for the amplification of *aac(3)-Ia*, *aac(3)-IIa*, and *aac(6′)-Ih* (Table 1, primer sets 1a to 1c, respectively). Triplex assay 2 included primers for the amplification of *aph(3′)-VI*, *ant(2″)-Ia*, and *rrn* (Table 1, primer sets 2a to 2c, respectively). Duplex assay 3 included primers for the amplification of *aph(3′)-Ia* and *aac(6′)-Ib* (Table 1, primer sets 3a and 3b, respectively). The amplification of *rrn*, a 16S rRNA gene fragment highly conserved among bacterial species, served as an internal control. The primer concentrations for *rrn* were reduced to improve the operating characteristics of the assay. Genomic DNA extracted from each bacterial isolate was used as the template. Primers developed from previously published sequences (27) were obtained commercially (Midland Certified Reagent Company, Midland, TX). Each reaction mixture contained 1× Eppendorf Master Mix (Eppendorf North America, Westbury, NY), 0.5 μl of 2.5 mM Mg^{2+}, 10 pmol of forward and reverse primers (0.5 pmol for the primers for *rrn*), and 20 ng of template DNA in a final volume of 25 μl. An initial denaturation step was carried out at 95°C for 5 min, followed by 30 cycles of amplification. Each cycle consisted of denaturation for 30 s at 95°C and annealing for 1 min at 49°C (triplex assays 1 and 2) or 54°C (duplex assay 3), followed by extension for 1 min at 72°C. The extension step was prolonged by 5 min for the final cycle. The reaction products were separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized by irradiation with UV light. Amplimers were identified by estimation of their lengths (in base pairs) by using a DNA ladder and were compared to the reaction products of the positive controls. Positive and negative controls were included with each reaction.

Predicted phenotypes. The expected phenotypes for aminoglycoside susceptibility were predicted from the genotype by the use of biochemical studies of substrate specificity and consensus reviews (5, 10, 14, 25, 26, 30, 38).

Aminoglycoside usage in the burn intensive care unit. The annual total usage of intravenous amikacin, gentamicin, and tobramycin in the burn intensive care unit from 2006 to 2008 was calculated from the dispensing records provided by the hospital pharmacy.

Statistical methods. The χ^2 test was used to examine the association of aminoglycoside resistance with genes encoding aminoglycoside-modifying enzymes.

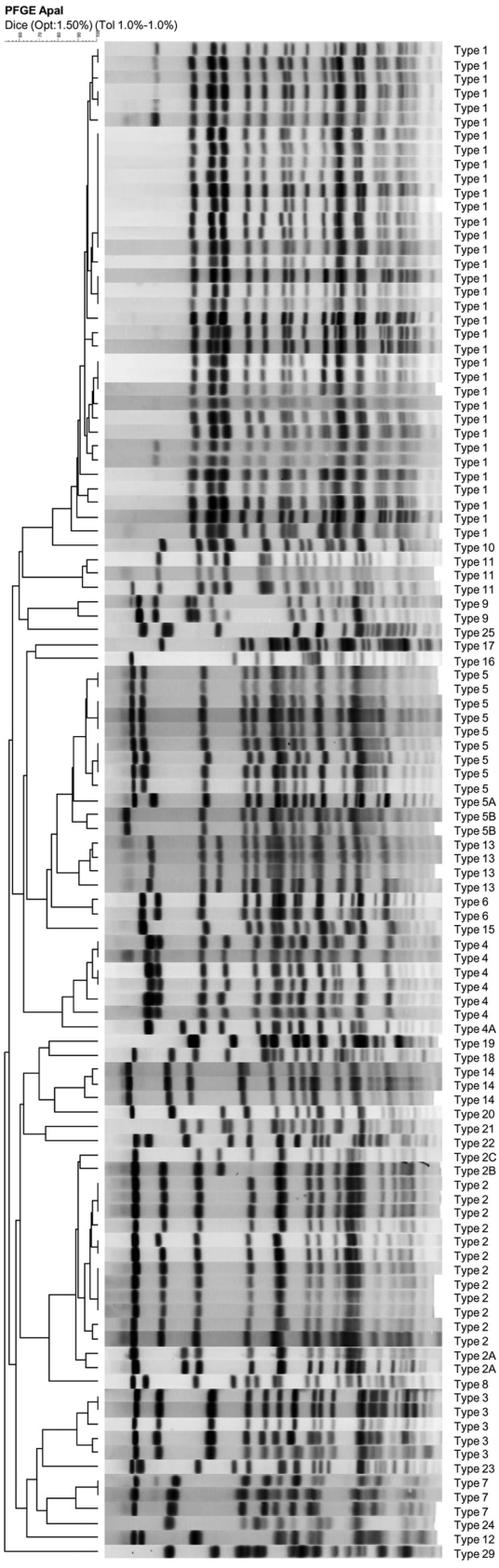

FIG. 1. PFGE patterns of 107 *Acinetobacter baumannii-calcoaceticus* complex isolates used in this study.

TABLE 2. MICs and error rates obtained for 107 isolates of the *Acinetobacter baumannii-calcoaceticus* complex tested for susceptibility to three aminoglycosides by six methods

Drug and method	MIC (μg/ml)		% S[a]	VME[b] (%)	ME[c] (%)	mE[d] (%)
	50%	90%				
Amikacin						
DD[e]			16.8	0.9	0	0
ET[f]	128	≥256	16.8	0.9	0	0
MicroScan	≥32	≥32	15.9	0.9	0.9	0
Phoenix	≥32	≥32	21.5	5.6	0	13.1
Vitek 2	≥16	≥64	53.3	36.4	0	13.1
BMD[g]	128	≥256	16.8			
Gentamicin						
DD			4.7	0	0	0
ET	≥256	≥256	4.7	0	0	0
MicroScan	≥8	≥8	4.7	0	0	0
Phoenix	≥8	≥8	5.6	0	0.9	0
Vitek 2	≥16	≥16	4.8	0	0	8.4
BMD	≥32	≥32	4.7			
Tobramycin						
DD			27.1	2.8	1.9	3.7
ET	24	≥64	28.0	2.8	1.9	0
MicroScan	≥8	≥8	27.1	1.9	2.8	4.7
Phoenix	≥8	≥8	30.8	5.6	0.9	4.7
Vitek 2	8	≥16	43.8	13.1	0.0	23.4
BMD	≥32	≥32	27.1			

[a] S, susceptible.
[b] VME, very major error.
[c] ME, major error.
[d] mE, minor error.
[e] DD, disk diffusion.
[f] ET, Etest.
[g] BMD, broth microdilution.

Statistical tests were performed with the SPSS program (version 16.0; SPSS Inc., Chicago, IL). A P value of <0.05 was considered statistically significant.

RESULTS

Characterization of clinical isolates. All isolates were identified by the clinical laboratory as members of the *A. baumannii-calcoaceticus* complex by use of the Vitek 2 instrument, and their identities were subsequently confirmed by use of the Phoenix instrument. In addition, isolates were characterized by various molecular methods. PFGE revealed 26 distinct clonal groups (Fig. 1). By the ARDRA method, 48 isolates were determined to be *A. baumannii* and 19 were determined to be *A. calcoaceticus*. The restriction enzyme digestion products of 40 isolates did not match those of *A. baumannii* or *A. calcoaceticus* reference strains, and thus, the strains could not be further resolved within the *A. baumannii-calcoaceticus* complex by these methods. However, the $bla_{\text{OXA-51-like}}$ beta-lactamase gene characteristic of *A. baumannii* was present in all the isolates.

Aminoglycoside susceptibility and error rates. The susceptibilities of the isolates to the aminoglycosides used at the San Antonio Military Medical Center were determined by multiple manual and automated methods, and the discordance of the results compared with those of the reference method (broth microdilution) was tabulated (Table 2). The activities of the aminoglycosides against the isolates were limited, with tobramycin being the most active agent tested. The levels of cate-

TABLE 3. Frequency of aminoglycoside-modifying enzyme genotypes, their predicted substrate specificity, number susceptible by the broth microdilution method, and correlation of PFGE types of 107 clinical isolates of *Acinetobacter baumannii-calcoaceticus* complex

Genotype	No. (%) of isolates	Subtrate(s)[a]	No. of isolates susceptible to[a]:			PFGE[b] type(s) (no. of isolates)
			GEN	TOB	AMK	
aph(3′)-Ia + ant(2″)-Ia	38 (35.5)	GEN, TOB	0	1	2	1 (31), 8 (1), 10 (1), 11 (3), 18 (1), 21 (1)
ant(2″)-Ia	23 (21.5)	GEN, TOB	0	0	1	2 (15), 3 (5), 17 (1), 23 (1), 25 (1)
aac(6′)-Ih + aph(3′)-Ia	11 (10.3)	TOB, AMK	0	9	9	4 (4), 6 (1), 7 (1), 13 (4), 22 (1)
aac(3)-Ia	9 (8.4)	GEN	3	7	3	5 (6), 16 (1), 24 (1), 29 (1)
aac(6′)-Ih	6 (5.6)	TOB, AMK	0	5	0	5 (6)
aac(3)-IIa	4 (3.7)	GEN, TOB	0	0	0	14 (3), 20 (1)
aac(3)-Ia + aph(3′)-Ia	4 (3.7)	GEN	0	2	2	1 (1), 4 (2), 6 (1)
aac(3)-Ia + ant(2″)-Ia	2 (1.9)	GEN, TOB	0	0	0	9 (2)
aac(3)-Ia + aph(3′)-Ia + aph(3′)-VI	2 (1.9)	GEN	0	1	0	7 (2)
aac(3)-Ia + aph(3′)-VI	1 (0.9)	GEN, AMK	0	1	0	4 (1)
aac(6′)-Ib + ant(2″)-Ia	1 (0.9)	GEN, TOB, AMK	0	0	0	12 (1)
aac(6′)-Ih + aph(3′)-VI	1 (0.9)	TOB, AMK	0	1	0	15 (1)
aph(3′)-Ia + aph(3′)-VI	1 (0.9)		1	1	0	1 (1)
aph(3′)-VI + ant(2″)-Ia	1 (0.9)	GEN, TOB, AMK	0	0	0	2 (1)
aac(3)-Ia + aph(3′)-Ia + ant(2″)-Ia	1 (0.9)	GEN, TOB	0	0	0	1 (1)
aac(6′)-Ih + aph(3′)-Ia + ant(2″)-Ia	1 (0.9)	GEN, TOB	0	0	0	1 (1)
None present	1 (0.9)		1	1	1	19 (1)

[a] GEN, gentamicin; TOB, tobramycin; AMK, amikacin.
[b] PFGE, pulsed-field gel electrophoresis.

gorical agreement of the results obtained by disk diffusion, by Etest, and with the MicroScan instrument with the results of broth microdilution were high for all three drugs. More manual testing errors occurred for tobramycin than for amikacin or gentamicin. High very major error and minor error rates were observed when susceptibility to amikacin and tobramycin was tested with the Phoenix and the Vitek 2 instruments.

Aminoglycoside-modifying enzymes. Aminoglycoside-modifying enzymes were inferred to be present by demonstration of the corresponding gene. All seven AME genes included in the multiplex PCR were present singly or in combination (Table 3). Forty-two isolates (39.3%) had one AME gene, 60 isolates (56.1%) had two AME genes, 4 isolates (3.7%) had three AME genes, and one pansusceptible isolate (0.9%) had no AME gene. The five most common AME genotypes were found in 87 of 107 isolates (81.3%), representing 19 (73.1%) of the 26 PFGE strains. The *ant(2″)-Ia* gene was statistically significantly associated with aminoglycoside resistance (χ^2 test, $P < 0.001$ for amikacin and tobramycin and $P = 0.013$ for gentamicin). Aminoglycoside resistance matched the predicted phenotype in 84 of 87 isolates (96.6%) for gentamicin, 69 of 89 isolates (77.5%) for tobramycin, and 12 of 21 isolates (57.1%) for amikacin. However, susceptibility to all three aminoglycosides matched the predicted phenotype in only nine isolates (8.4%). Susceptibility was retained in the presence of a gene for a potentially inactivating AME in 3 isolates (3.4%) for gentamicin, 16 isolates (18.0%) for tobramycin, and 9 isolates (42.9%) for amikacin.

Influence of annual aminoglycoside usage on phenotypes and genotypes. To determine the effect of aminoglycoside use on resistance rates and AME gene prevalence, a subset of 45 isolates from the burn intensive care unit were compared by year of occurrence and annual aminoglycoside use in that unit (Table 4). The rate of amikacin use greatly exceeded that of tobramycin or gentamicin. The susceptibilities of the isolates matched those predicted by the AME genotype for 36 of 36 isolates (100%) for gentamicin, 29 of 37 isolates (78.4%) for tobramycin, and 6 of 8 isolates (75%) for amikacin (Table 5). In contrast, genotypes and phenotypes were matched for all three aminoglycosides in only 2 of 45 isolates (4.4%). Susceptibility was retained in the presence of a potentially inactivating AME gene in eight isolates (21.6%) for tobramycin and two isolates (25.0%) for amikacin. All isolates from the burn ICU having a gentamicin-inactivating AME gene were resistant to gentamicin. The incidence of tobramycin susceptibility among the burn ICU isolates increased over the 3-year period, but this did not correlate with convergence toward a single strain or the absence of a tobramycin resistance gene. In fact, tobramycin-inactivating AME genes were found in all strains from 2008, when the rate of tobramycin susceptibility was the greatest. Multiple PFGE

TABLE 4. Total annual aminoglycoside usage in the burn intensive care unit

Yr	Amikacin		Tobramycin		Gentamicin	
	Total annual usage (g)	No. (%) of isolates susceptible	Total annual usage (g)	No. (%) of isolates susceptible	Total annual usage (g)	No. (%) of isolates susceptible
2006	326.4	1 (9.1)	15.0	1 (9.1)	15.3	0 (0)
2007	514.9	0 (0)	1.3	5 (23.8)	7.0	1 (4.8)
2008	506.3	3 (23.1)	19.4	9 (69.2)	0	0 (0)

TABLE 5. Susceptibility, prevalence of aminoglycoside-modifying enzyme genotypes, and clonal diversity of 45 isolates of *A. baumannii-calcoaceticus* complex from burn intensive care unit

Genotype	Substrate(s)[a]	PFGE type (no. of isolates)		
		2006	2007	2008
ant(2″)-Ia	GEN, TOB	3 (2)	2 (3)	
aac(3)-Ia	GEN		5 (4)	5 (1)
aac(6′)-Ih	TOB, AMK			5 (5)
aac(3)-IIa	GEN, TOB			14 (2)
aac(3)-Ia + aph(3′)-Ia	GEN	4 (1)		
aac(3)-Ia + ant(2″)-Ia	GEN, TOB		9 (1)	
aac(6′)-Ih + aph(3′)-Ia	TOB, AMK			7 (1), 13 (2)
aph(3′)-Ia + aph(3′)-VI			1 (1)	
aph(3′)-Ia + ant(2″)-Ia	GEN, TOB	1 (7), 11 (1)	1 (10)	
aph(3′)-VI + ant(2″)-Ia	GEN, TOB, AMK			1 (1), 11 (1)
aac(3)-Ia + aph(3′)-Ia + aph(3′)-VI	GEN		7 (1)	
aac(3)-Ia + aph(3′)-Ia + ant(2″)-Ia	GEN, TOB		1 (1)	

[a] GEN, gentamicin; TOB, tobramycin; AMK, amikacin.

types were observed across all 3 years, and no exclusive relationships between PFGE type and AME genes were identified.

DISCUSSION

Automated susceptibility testing methods are appealing on the basis of efficiency, convenience, and the time to the retrieval of the results and are widely used in modern clinical microbiology laboratories. Our data highlight the limitations of both the automated and the manual testing methods, the inaccuracies of which may further restrict the shrinking number of agents useful against multidrug-resistant bacteria or encourage treatment with inactive agents. In addition, we found a diversity of AMEs across heterogeneous strains of the *A. baumannii-calcoaceticus* complex which did not correlate with aminoglycoside susceptibility or prescribing patterns.

Significant errors in aminoglycoside susceptibility were observed across multiple testing methods in this study. The Phoenix and MicroScan instruments were more accurate than the Vitek 2 instrument, but the very major error rates for amikacin and tobramycin in all three automated systems approached or exceeded the levels allowable by the CLSI (35). These errors did not appear to correlate with specific aminoglycoside-modifying enzymes. In addition to amikacin, for which confirmation of the Vitek 2 system results by use of a manual method is recommended (3), we also observed high very major error rates for tobramycin by all three automated methods. Although the errors for gentamicin were within the established limits, interpretation was limited because of the lack of isolates with MIC values surrounding the CLSI breakpoints. Disk diffusion and Etest performed as well as or better than the automated methods, but excess very major errors also occurred with manual tobramycin testing. We therefore recommend broth microdilution as the most accurate method for determining the aminoglycoside susceptibilities of isolates in the *A. baumannii-calcoaceticus* complex. However, many laboratories may lack the ability to perform this technically complex, time-consuming, and labor-intensive method.

Various studies have examined the accuracies of automated platforms for determination of the aminoglycoside susceptibilities of nonfermenting Gram-negative bacilli (16, 17, 21, 22, 24, 31), but few have reported data specifically for *A. baumannii*. For 20 isolates from Taiwan, the data obtained by use of the Vitek 2 instrument and the AST-GN10 card agreed fully with the data obtained by the agar dilution method for amikacin, gentamicin, and tobramycin (16). For 25 isolates from Spain tested with the Vitek 2 instrument and the AST-GN11 card, higher minor error rates were observed for tobramycin (12%) than for gentamicin (4%), and there were no very major or major errors; amikacin was not tested (17). In contrast to the findings of those studies, a very major error rate of 83.1% for amikacin with the Vitek 2 system and the AST-GN9 and AST-GN59 cards was recently reported for 116 isolates from Israel (15). A study of mixed isolates from Germany and Italy included 30 non-*Pseudomonas*, nonfermenting Gram-negative bacilli, 10 of which were *A. baumannii*. For these mixed isolates, the Phoenix instrument demonstrated very major error rates of 5.9% for amikacin, 4.8% for tobramycin, and 0% for gentamicin. A very major error rate of 11.1% for amikacin was noted among 79 *Pseudomonas aeruginosa* isolates (24), indicating that the scope of this problem extends to multiple bacterial species and susceptibility testing platforms.

A wide array of aminoglycoside-modifying enzymes have previously been reported in *A. baumannii*. The largest published data set evaluated AMEs detected by a combination of phenotypic inference and DNA hybridization in 1,189 *Acinetobacter* sp. isolates from South Africa, Europe, China, Latin America, and Mediterranean countries (25). The predominant AME was an AAC(3)-class enzyme, which occurred in nearly 50% of the isolates. Next, in order of prevalence, were the combinations of AAC(3) plus ANT(2″)-I and APH(3′)-VI plus ANT (2″)-I, which accounted for 11.3% of AMEs. A high overall incidence of APH(3′)-VI (46.2%) was reported. More recently, a South Korean study reported a different prevalence of AME genes in a polyclonal group of *A. baumannii* isolates: *aac(3)-Ia* in 14.8%, *aac(6′)-Ib* in 83.6%, *ant(3″)-Ia* in 85.2%, *aph(3′)-Ia* in 88.5%, and *aph(3′)-VI* in 1.6% (9). In contrast, we found AMEs that acetylate to be less common (42.6%) among our isolates. The *ant(2″)-Ia* adenylase was the predominant gene present in our isolates (62.6%) and the only one statistically correlated with resistance to each of the aminoglycosides tested. Interestingly, this included resistance to amikacin, which is not a favorable substrate for ANT(2″)-Ia (14). The

next most common gene was *aph(3′)-Ia* (54.2%), for which kanamycin is a better substrate than gentamicin, tobramycin, or amikacin (30). This gene is thus of less clinical importance in the San Antonio Military Medical Center, where kanamycin is not used. The level of correlation between the PFGE type and the genotype was low, possibly due to the frequent importation of new strains into the San Antonio Military Medical Center (28).

We observed frequent disagreement between the predicted and the actual aminoglycoside phenotypes, with the phenotypes for only 8.4% being in full agreement. This suggests that other aminoglycoside resistance mechanisms known in this species, potentially including reduced permeability, efflux pumps, 16S rRNA methylation, and other modifying enzymes, may be active. We have previously found the multidrug efflux transporter AdeABC (known to extrude aminoglycosides) in 98% of our isolates (data not shown). Although broad-spectrum inhibitors of AMEs have been discovered and proposed for use in combination with aminoglycosides, following the paradigm of the beta-lactamase inhibitors (4), a therapeutic strategy addressing a single mechanism is unlikely to be successful without the concurrent inhibition of all active resistance mechanisms.

Antibiotic pressure has been correlated with the development of aminoglycoside resistance (7, 20). In our burn intensive care unit, where aminoglycoside resistance is prevalent, amikacin was used in greater quantities than gentamicin and tobramycin. While high rates of amikacin resistance in *Acinetobacter* may not be surprising, this did not reflect a convergence toward particular strains or aminoglycoside-modifying enzymes. Likewise, the incidence of tobramycin susceptibility increased over the 3-year period, despite the presence of tobramycin-inactivating AME genes in all of the susceptible isolates. In contrast, gentamicin resistance was nearly universal, even though it was the least-used aminoglycoside overall. These discrepancies suggest that aminoglycoside resistance in *A. baumannii* is mediated by complex and multifactorial mechanisms.

In conclusion, modern automated susceptibility testing platforms as well as manual methods are vulnerable to errors when isolates of *A. baumannii-calcoaceticus* complex are tested for their aminoglycoside susceptibilities, and the use of broth microdilution testing may be advisable to optimize the accuracy of the susceptibility testing results. Such errors may jeopardize clinical care by encouraging treatment with inactive agents or falsely restricting the available therapeutic options. These errors do not appear to be attributable to the AME genes carried by the organism. As in earlier reports, the composition of AME genes in contemporary isolates of the *A. baumannii-calcoaceticus* complex is diverse, but it appears to differ from that indicated in earlier reports. Aminoglycoside resistance at the San Antonio Military Medical Center does not appear to be caused by the clonal expansion of isolates containing characteristic AME genotypes, nor does aminoglycoside usage appear to be directly propelling resistance to this class among the *A. baumannii-calcoaceticus* complex isolates in the burn intensive care unit. For most isolates, the AME genotype was an inadequate predictor of the aminoglycoside phenotype, suggesting that a complex amalgam of multiple resistance mechanisms are operating simultaneously but have variable expression. Determining the relative contribution of multiple concurrent resistance mechanisms may improve our understanding of aminoglycoside resistance in the *Acinetobacter baumannii-calcoaceticus* complex.

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